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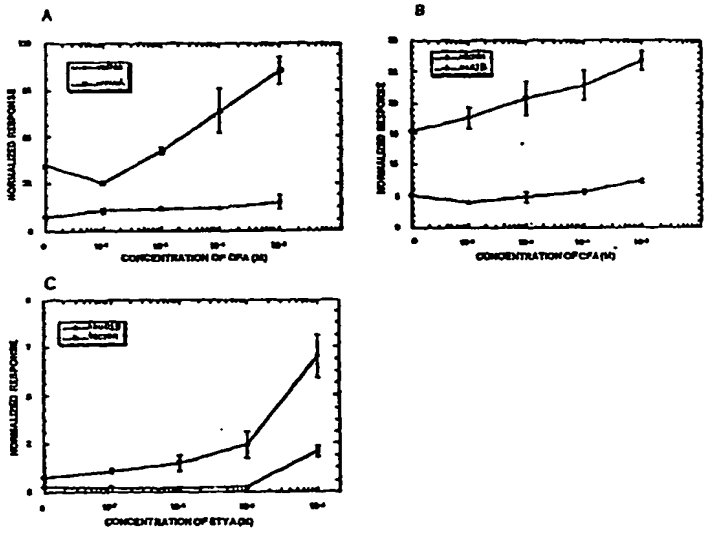

(57) Abstract

This invention provides methods for screening for agents useful for treatment of diseases and pathological conditions affected by the level of NUC protein activity. These agents reduce or relieve the repression of PPARα protein and TR protein transcription activation activity by NUC protein. The selected novel or unique agents can be used to treat hyperlipidemia, hypercholesteremia and hyperlipoproteinemia.

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DESCRIPTION

SCREENING FOR NUC INHIBITORS

Cross Reference to Related Applications

This application is related to U.S. Application Serial No. 08/143,215, titled "Human Peroxisome Proliferator Activated Receptor," filed October 25, 1993, by Mukherjee, which is a continuation-in-part of Application Serial No. 08/141,500, titled "Human Peroxisome Proliferator Activated Receptor," filed October 22, 1993, by Mukherjee; the disclosures of which are incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to screening for agents active on peroxisome proliferator activated receptor (PPAR) and thyroid hormone receptor (TR) related molecules. This invention also relates to the cloning and sequencing of a new human peroxisome proliferator activated receptor named hNUC1B.

BACKGROUND OF THE INVENTION

Peroxisomes are subcellular organelles found in animals and plants. Peroxisomes contain enzymes for cholesterol and lipid metabolism and respiration.

A variety of chemical agents called peroxisome proliferators induce the proliferation of peroxisomes. Peroxisome proliferators include unsaturated fatty acids, hypolipidemic drugs (Reddy, J. K., and Azarnoff, D. L. (1980) Nature **283**, 397-398), herbicides, leukotriene antagonists, and plasticizers (for a review, see Green, S., Biochem. Pharmacol. 43:393-400, 1992). Hypolipidemic drugs such as clofibrates have been found to lower triglycerides and cholesterol levels in plasma and to be beneficial in the prevention of ischemic heart disease in individuals with elevated levels of cholesterol (Havel, R.J. and Kane, J.P., Ann. Rev. Pharmac. 13:287-308, 1973).

Therapeutic use of such drugs, however, is questioned because clofibrates are carcinogens in rats.

There are two hypotheses for peroxisome proliferation. The "lipid overload hypothesis" suggests that an increase in the intracellular concentration of fatty acids is the main stimulus for peroxisome proliferation (Nestel, P. J. (1990) Ann. Rev. Nutr. **10**, 149-167 and Phillipson, B. E., Rothrock, D. W., Connor, W. E., Harris, W. S., and Illingworth, D. R. (1985) N. Engld. J. Med. **312**, 1210-1216). Another hypothesis postulates a receptor mediated mechanism. Peroxisome proliferator activated receptors (PPARs) have been isolated and cloned from various species (Isseman, I., and Green, S. (1990) Nature **347**, 645-650; Dreyer, C., Krey, G., Hansjorg, K., Givel, F., Helftenbein, G., and Wahli, W. (1992) Cell **68**, 879-887; Gottlicher, M., Widmar, E., Li, Q., and Gustafsson, J. A. (1992) Proc. Natl. Acad. Sci. USA. **89**, 4653-4657; Sher, T., Yi, H. F., McBride, W. O. and Gonzales., F. J. (1993) Biochemistry **32**, 5598-5604; and Schmidt, A., N. Endo, S. J. Rutledge, R. Vogel, D. Shinar, and G. A. Rodan. (1992) Mol. Endocrinol. **6**, 1634-16414-8). The ligand for PPARs is still unidentified.

Issemann and Green, Nature 347:645-650, 1990, cloned a mouse peroxisome proliferator activated receptor (mPPAR) gene from a mouse liver complementary DNA (cDNA) library. Göttlicher et al., Proc. Nat. Acad. Sci. USA 89:4653-4657, 1992, cloned a rat peroxisome proliferator activated receptor (rPPAR) gene from a rat liver cDNA library. PPARs from mouse and rat share 97% homology in amino acid sequence and a particularly well-conserved putative ligand-binding domain. Three members of the Xenopus nuclear hormone receptor superfamily (i.e., XPPARα, XPPARβ and XPPARγ) have also been found to be structurally and functionally related to the mPPAR (Dreyer et al., Cell 68:879-887, 1992).

Schmidt et al., Molecular Endocrinology 6:1634-1641,

1992, cloned a steroid hormone receptor gene, hNUC1, from a human osteosarcoma cell cDNA library. The homology between amino acid sequence of hNUC1 and that of mPPAR is 62%.

Sher et al., Biochemistry 32:5598-5604, 1993, cloned a human PPAR gene from a human liver cDNA library. This clone has 85% nucleotide sequence homology and 91% amino acid sequence homology with the mPPAR clone.

Peroxisome proliferator activated receptor (PPAR) is a member of the steroid receptor family. PPAR is divided into several subfamilies based upon their primary sequence homology. Fang et al., Biochem. Biophy. Res. Com. 196:671-677, 1993 discussed XPPARα, XPPARβ, XPPARγ, mPPAR and hNUC1.

SUMMARY OF THE INVENTION

This invention relates to the cloning, sequencing and expression of a human peroxisome proliferator activated receptor subtype, hNUC1B. hNUC1B differs in amino acid sequence from hNUC1 by one amino acid (i.e., alanine at position 292).

Applicant has determined that hNUC1B protein represses hPPARα (hPPARα, referred to as hPPAR1 in U.S. Application Serial No. 08/143,215, is a subtype of PPAR) and TR protein activity, and that relief from such repression is therapeutically useful.

Thus, the present invention features methods for identifying therapeutic agents that alleviate the repressive effects of NUC protein on PPARα protein and TR protein activity and for using these agents to treat diseases and pathological conditions affected by the level of NUC protein activity, such as, but not limited to, hyperlipidemia, hypercholesteremia and hyperlipoproteinemia. These methods make it possible to screen large collections of natural, semisynthetic, or synthetic compounds for therapeutic agents that relieve the repression of PPARα or TR activity by NUC protein.

By "PPARα protein" is meant a PPAR subtype protein that is substantially homologous (i.e., no less than 90% homologous in amino acid sequence) to hPPARα protein, including, but not limited to, hPPARα.

By "NUC protein" is meant a PPAR subtype protein or a PPAR related protein that represses the transcription activation activity of PPARα protein and/or TR protein, including, but not limited to, hNUC1B protein, hNUC1 protein and proteins with homologous sequences to hNUC1B or hNUC1 protein.

This invention is also directed to compounds, compositions, and methods for modulating processes affected by NUC protein activity and useful for treating a patient exhibiting a pathological condition caused, induced or aggravated by the level of NUC protein activity. More particularly, the invention relates to compounds and pharmaceutical compositions that relieve the repression of PPARα protein and TR protein activity by a NUC protein.

Thus, in one aspect, the present invention features a method for identifying therapeutic agents for treatment of a pathological condition affected by the level of NUC protein activity, comprising the step of screening for an inhibitor of NUC protein activity.

One method comprises identifying therapeutic agents which, when added to a system containing NUC protein and PPARα protein, relieve the repression of PPARα protein activity by NUC protein.

In a preferred embodiment, this system further contains a reporter gene responsive to PPARα protein activation, the reduction or relief of the repression of PPARα by NUC protein is measured by the expression level of the reporter gene.

By "reporter gene" is meant a gene encoding a product that is easily detected and assayed by techniques known to those skilled in the art. A reporter gene in this invention is driven by a promoter that is responsive

to PPARα protein or TR protein, including, but not limited to, the native promoter of a gene such as acylcoenzyme A oxidase, enoyl-CoA hydratase/3-hydrosyacyl-CoA dehydrogenase bifunctional enzyme or 3-ketoacyl thiolase.

In a further preferred embodiment, the screening assay is conducted in a cell.

In an even further preferred embodiment, NUC gene, PPARα gene and the reporter gene are encoded in vectors and introduced into the cell by transfection. The reporter gene has a peroxisome proliferator responsive element (PPRE) and can be activated by PPARα protein.

In another further preferred embodiment, the screening assay is conducted in an extract of a cell by in vitro transcription.

In a third further preferred embodiment, a PPAR activator is added to the screening assay.

By "PPAR activator" is meant a chemical agent that is capable of activating the transcription activation activity of PPARα protein, such as, but not limited to, CFA (clofibric acid), ETYA (5,8,11,14-eicosatetraynoic acid) or WY-14, 643 ([4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid).

Another method comprises identifying therapeutic agents which, when added to a system containing NUC protein and TR protein, relieve the repression of TR protein activity by NUC protein.

In a preferred embodiment, this system further contains a reporter gene responsive to TR protein activation, the repression or relief of the repression of TR by NUC is measured by the expression level of the reporter gene.

In a further preferred embodiment, the screening assay is conducted in a cell.

In an even further preferred embodiment, NUC gene, TR gene and the reporter gene are encoded in vectors and introduced into the cell by transfection. The reporter gene has a thyroid hormone responsive element (TRE) and

can be activated by TR protein. TREs include, but are not limited to, TREp (palindromic TRE) and DR4 (direct repeat with a 4 nucleotide spacing 5'-AGGTCACAGGAGGTCA-3').

In another further preferred embodiment, the screening assay is conducted in an extract of a cell by in vitro transcription.

In a third further preferred embodiment, a TR activator is added to the screening assay to activate TR.

By "TR activator" is meant a chemical agent that is capable of activating the transcription activation activity of TR protein, such as, but not limited to, LT3 (3,3',5-triiodo-L-thyronine), LT4 (L-thyroxine) or Triac (3,3',5-triiodothyroacetic acid).

A third method comprises identifying agents which, when added to a system containing a NUC protein and a nucleic acid (such as an oligonucleotide) including a PPRE, reduce the binding of NUC protein to the nucleic acid. The level of binding can be detected in a gel retardation assay or other assays known to those skilled in the art.

A fourth method comprises identifying agents which, when added to a system containing a NUC protein and a PPARα protein, reduce the formation of NUC-PPARα complexes.

In a preferred embodiment, NUC protein is labeled and the formation of NUC-PPARα complexes is measured by the amount of labeled NUC protein precipitated by PPARα specific antibody.

A fifth method comprises identifying agents which, when added to a system containing a NUC protein and a TR protein, reduce the formation of NUC-TR complexes.

In a preferred embodiment, NUC protein is labeled and the formation of NUC-TR complexes is measured by the amount of labeled NUC protein precipitated by TR specific antibody.

In another aspect, this invention features a method for treatment of a pathological condition affected by the

level of NUC protein activity by providing an agent that represses or reduces the NUC protein activity. The pathological conditions treated by this method include, but are not limited to, hyperlipidemia, hypercholesteremia and hyperlipoproteinemia.

This invention also relates to novel or unique therapeutic agents discovered by the above methods, i.e., agents that are not known per se or agents that are not already known for use related to treatment of a pathological condition affected by the level of NUC protein activity.

Applicant is particularly interested in the identification of agents of low molecular weight (less than 10,000 daltons, preferably less than 5,000, and most preferably less than 1,000) which can be readily formulated as useful therapeutic agents.

Such agents can then be screened to ensure that they are specific to tissues with pathological conditions induced or aggravated by NUC protein with little or no effect on healthy tissues such that the agents can be used in a therapeutic or prophylactic manner. If such agents have some effect on healthy tissues they may still be useful in therapeutic treatment, particularly in those diseases which are life threatening.

The therapeutic agents discovered by the above assays can then be screened for tissue specificity and toxicity with methods known to those skilled in the art. They can be put in pharmaceutically acceptable formulations, and used for treatment of diseases and pathological conditions induced or aggravated by NUC protein activity.

Once identified, a NUC inhibitor can be used to study the mechanism of NUC inhibition of PPARα and TR protein activity. Applying techniques known to those skilled in the art, such as those described in J. Sambrook, E. F. Fritsch, and T. Maniatis, Molecular Cloning: A Laboratory Manual, 2 Ed., Cold Spring Harbor

Laboratory Press, Cold Spring Harbor, New York, 1989, a NUC inhibitor can also be used to study the structural changes in NUC protein when the inhibitor binds to the NUC protein, and how the binding affects the ability of the NUC protein to interact with other proteins and to bind to DNA.

Other features and advantages of the invention will be apparent from the following detailed description of the invention, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (A-C) is a graph showing normalized luciferase activity. HepG2 cells were transfected with (A) vector pCMVhPPARα, or pBKCMV (see "Vector construction" in the detailed description of the invention) and treated with CFA. Cells were transfected with pCMVhNUC1B (see "Vector construction" in the detailed description of the invention) or vector and treated with CFA (B) or ETYA (C) and luciferase and β-galactosidase assays performed as described in materials and methods.

Figure 2 is a graph showing normalized response of reporter gene to increasing dose of hNUC1B. HepG2 cells were transfected with 0.1 μg pCMVhPPARα and increasing amounts of pCMVhNUC1B (indicated in μgs). CFA was added to a final concentration of 1 mM. Control cells received an equal volume of ethanol (vehicle).

Figure 3 (A-E) is a graph showing normalized response of various receptors to hNUC1B. HepG2 cells were transfected with (A) the ER expression vector HEO (1μg) and pLPwtCAT (see "Vector construction" in the detailed description of the invention) (1.5 μg) reporter (B) pRShRARα (see "Vector construction" in the detailed description of the invention) and MTV-TREp2-LUC (C) pRShRARα and CRBPII-tk-LUC (D) pRShTRβ (see "Vector construction" in the detailed description of the invention) and MTV-TREp2-LUC. 0.1 μg of expression plasmid and 0.5 μg luciferase reporter were used (B-D).

pCMVhNUC1B was co-transfected where indicated, 1μg (A) and 0.1 μg (B-D). The respective ligands for the transfected receptors were β-estradiol (100nM), ATRA (all-*trans*-retinoic acid) (1 μM), 9-*cis*-RA (1 μM) and L-T3 (100 nM), the final concentration indicated within parentheses. CFA was added where indicated to a final concentration of 1 mM. In Figure 3E, transfections were done as in Figure 3D except that the reporter gene had two copies of DR4 element (direct repeat separated by four nucleotides). The sequence of the DR4 element used is 5'-AGGTCACAGGAGGTCA-3'. The repeat sequence is shown with a line above it.

Figure 4 is a graph showing normalized response of hPPARα to hNUC1B with increasing ratio of hPPARα to hNUC1B. HepG2 cells were transfected with 0.05 μg of pCMVhNUC1B and different amounts of pCMVhPPARα plasmid (indicated in μgs). CFA was added to a final concentration of 1 mM.

Figure 5 is a radiograph of a gel retardation assay. DNA binding assays were performed with extracts from COS cells transfected with pCMVhNUC1B or pRShRXRα as described in materials and methods. Extracts from mock transfected cells were used as a control.

DETAILED DESCRIPTION OF THE INVENTION

NUC Proteins and Peroxisome Proliferation

The effect of hypolipidemic drugs like gemfibrozil that have significant cardio-protective effect are mediated via the PPARs. To investigate the effect of various fibrates on human PPAR subtypes, we have isolated two human PPAR subtypes, i.e., PPARα and hNUC1B. Applicant determined that hNUC1B is not activated by PPAR activators such as clofibric acid. Applicant also determined that hNUC1B is a specific repressor of the transcriptional activation effected by PPARα and thyroid hormone receptor. The repressive action of NUC protein on PPARα and TR receptors may limit the clinical efficacy of

PPARα and TR activators (e.g., fibrates, synthroid). Agents that relieve this repression will increase activity of PPARα and TR and increase the efficacy of existing drugs, or render these drugs unnecessary.

A subtype of NUC protein, hNUC1, has been shown to be present in the human heart, brain, and liver tissues where PPARs and TRs are active. Therefore, the screening methods of this invention and agents identified thereby may have widespread therapeutic significance.

We have demonstrated co-operative binding of hNUC1B and RXRα to a PPAR response element, PPRE. Without being bound by any particular theory, applicant proposes that repression of PPARα by hNUC1B likely occurs at the level of competition for DNA binding, or titrating factors required for PPARα and TR activity. hNUC1B could be binding to the PPRE, thereby antagonizing activation of PPARα protein. COUP-TF (chicken ovalbumin upstream promoter transcription factor) has been shown to inhibit PPAR activation by a similar mechanism (Miyata, K.S., Zhang, B., Marcus, S.L., Capone, J.P., and Rachubinski, R.A. (1993) Journ. Biol. Chem. **268**, 19169-19172). In the absence of a transcription activation function of hNUC1B, this mechanism could explain the repression of PPARα activity by hNUC1B.

The invention will now be described in greater detail by reference to the following examples regarding screening for NUC inhibitors. This invention, however, is not limited to co-transfection assay, gel retardation assay and immunoprecipitation assay described below. Other methods known to those skilled in the art for assaying an agent that relieve the repressive effect of a protein on a cellular activity may also be used.

Examples

A candidate agent can be screened by either A) indirect evaluation of derepression of a PPARα or TR responsive gene, B) direct evaluation of NUC protein

binding to a PPARα or TR responsive element, or C) direct evaluation of complex formation between NUC protein and PPARα protein or TR protein.

Experimental procedures and reagents employed in the examples described herein are set forth below:

Reagents

ETYA, β-estradiol, ATRA, LT3 (3,3',5-triiodo-L-thyronine) and CFA were purchased from Sigma, and WY-14,643 from Chemsyn Science Laboratories, Lenexa, Kansas, USA. Stock solutions of these compounds were made in ethanol, methanol or dimethyl sulfoxide.

The recipes for buffers, mediums, and solutions in the following examples are given in J. Sambrook, E. F. Fritsch, and T. Maniatis, Molecular Cloning: A Laboratory Manual, 2 Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.

Vector Construction

For expression in mammalian cells, the hPPARα cDNA was cloned into the NotI site of pBKCMV (Stratagene) to give pCMVhPPARα.

The hNUC1B cDNA was directionally cloned into the SalI-SacII site of pBKCMV to give pCMVhNUC1B.

The reporter plasmid pPPREA3-tk-luc was generated by inserting three copies of the synthetic oligonucleotide (5'-CCCGAACGTGACCTTTGTCCTGGTCC-3') containing the "A" site of the Acyl-CoA oxidase gene regulatory sequence (Osumi, T., Wen, J. and Hashimoto, T. (1991) Biochem. Biophys. Res. Commun. **175**, 866-871) into the XhoI site 5' of the tk promoter in the previously described pBLtk-luciferase vector (Giguere, V., Hollenberg, S. M., Rosenfeld, M. G., Evans, R. M. (1986) Cell **46**, 645-652).

pRShRARα, pRShRXRα, MTV-TREp2-LUC, and CRBPII-tk-LUC have been described in Giguere,V., Ong, E.S., Segui, P., and Evans, R.M. (1987) Nature **330**(2), 624-629; Mangelsdorf, D.J., Ong, E.S., Dyck, J.A., and Evans, R.M.

(1990) Nature 345, 224-229; Umesono, K., Giguere, V., Glass, C.K., and Rosenfeld, M.G. (1988) Nature 336, 262-265 and Mangelsdorf, D.J., Umesono, K., Kleiwer, S.A., Borgmeyer, U., Ong, E.S., and Evans, R.M. (1991) Cell 66, 555-561.

pRShTRβ has been described in Thomson, C.C., and Evans, R.M. (1989) Proc. Natl. Acad. Sci. USA. 86, 3494-3498.

The human TRα1 cDNA (Nakai, A., Sakurai, A., Bell, G.I., and DeGroot, L.J. (1988) Molec. Endoc. 2, 1087-1092) was liberated from pME21 by digestion with EcoR1 and blunt ended by digestion with mung bean nuclease. pRS plasmid (Giguere, V., Hollenberg, S. M., Rosenfeld, M. G., Evans, R. M. (1986) Cell 46, 645-652) was digested with BamH1, dephosphorylated and repaired with Klenow enzyme. The TRα1 cDNA was then joined to the vector by blunt end ligation.

The ER expression plasmid HEO has been described in (Kumar, V. and Chambon, P. (1988) Cell 55, 145-156).

The estrogen inducible brain creatine kinase promoter was cloned into pUCPLCAT to give pLPwtCAT.

Co-transfection Assay

HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%(v/v) fetal bovine serum (Hyclone), 2 mM L-glutamine, and 55 μg/ml gentamicin (BioWhittaker). Cells were plated at 2 x 10^5 cells per well for HepG2 in 12 well cell culture dishes (Costar). The media was replaced with fresh media 20 hours later. After 4 hours, DNA was added by the calcium phosphate coprecipitation technique (Berger, T. S., Parandosh, Z., Perry, B., and Stein, R.B. (1992) J. Steroid. Biochem. Molec. Biol. 41, 733-738). Typically, 0.1 μg of expression plasmid, 0.5 μg of the β-gal expression plasmid pCH110 (internal control), and 0.5 mg of reporter plasmid were added to each well.

Where indicated, 0-0.5 μg of hNUC1B plasmid

(repressor) was added. Repressor plasmid dosage was kept constant by the addition of appropriate amounts of the empty expression vector pBKCMV. Total amount of DNA was kept at 20 μg by the addition of pGEM DNA (Promega).

After 14 hours the cells were washed with 1X PBS and fresh media added (DMEM with 10% charcoal stripped fetal bovine serum (Hyclone) plus the above supplements). Ligands or PPAR activators were added to the final concentrations indicated. Control cells were treated with vehicle.

After another 24 hours the cells were harvested and the luciferase and β-galactosidase activities quantified on a Dynatech ML 1000 luminometer and a Beckman Biomek 1000 workstation respectively. The normalized response is the luciferase activity of the extract divided by the β-galactosidase activity of the same. Each data point represents the mean of three transfections. Error bars represent the standard deviation from the mean. CAT assays were performed as in Ausbel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1987) in Current Protocols in Molecular Biology, Wiley Interscience.

Gel Retardation Assay

COS cells were transfected with 5 μg of pCMVhNUC1B or pRShRXRα (Ptashne, M. (1988) Nature 335, 683-689) per 100 mm dish for 48 hours. Whole cell extracts were made by four cycles of freeze-thawing in 0.4 M KCl containing buffer followed by centrifugation. Gel retardations were performed by incubating 5 μg of cell extract in buffer containing 10 mM Hepes (7.8), 50 mM KCl, 1 mM DTT, 2.5 mM $MgCl_2$, 0.5mg/ml dIdC and 20% glycerol at 4°C for 5 minutes. About 100,000 cpm of ^{32}P-end-labeled probe was then added and incubated at 25°C for another 5 minutes.

Protein-DNA complexes were resolved by electrophoresis on 5% polyacrylamide gels in 0.5X TBE. The PPRE sequence from the acyl-coenzymeA oxidase (AOX)

gene used as probe is
5'-CTAGCGATATCATGACCTTTGTCCTAGGCCTC-3' (upper strand) and
5'-CTAGGAGGCCTAGGACAAAGGTCATGATATCG-3' (lower strand).

Example 1. Cloning of hPPARα

A human homologue of rat PPARα was isolated from a human liver 5'-stretch lgt10 cDNA library (Clontech). The library was screened at medium stringency (40% formamide, 5X SSC at 37°C), with a rPPAR nick translated DNA fragment specific to the A/B and DNA binding domain (from the EcoR1 to the BglII site, nucleotides 450-909) (Gottlicher, M., Widmar, E., Li, Q., and Gustafsson, J. A. (1992) Proc. Natl. Acad. Sci. USA. **89**, 4653-4657). Positive clones were isolated and subcloned into the Bluescript KS vector (Stratagene) for sequencing. The sequence is identical to that published by Sher et. al., (Sher, T., Yi, H. F., McBride, W. O. and Gonzales., F. J. (1993) Biochemistry **32**, 5598-5604) except for two amino acid differences, alanine at position 268 and glycine at position 296.

The activation profile of hPPARα by CFA is shown in Fig. 1A. This receptor is also activated by other known activators of PPARs, e.g., WY-14,643 and ETYA in HepG2 and CV-1 cells.

Example 2. Cloning of hNUC1B

hNUC1B was isolated from a human kidney cDNA library by screening with a probe specific to the rat PPAR DNA binding domain (from the PvuII to the BglII site, nucleotides 618-909, reference (Gottlicher, M., Widmar, E., Li, Q., and Gustafsson, J. A. (1992) Proc. Natl. Acad. Sci. USA. **89**, 4653-4657)) using procedures as described above in Example 1. A recombinant clone was isolated, subcloned into pGEM-5Zf and sequenced. The sequence of this receptor is identical to that of the hNUC1 sequence (Schmidt, A., N. Endo, S. J. Rutledge, R. Vogel, D. Shinar, and G. A. Rodan. (1992) Mol. Endocrinol. **6**, 1634-1641) except for alanine at position 292.

hNUC1B is a member of the PPAR family. hNUC1B has 61% homology to hPPARα and the two cysteine residues in the "D" box are separated by three amino acids (E, R and S, positions 112-114 of the amino acid sequence). This is a characteristic of PPARs (Dreyer, C., Krey, G., Hansjorg, K., Givel, F., Helftenbein, G., and Wahli, W. (1992) Cell 68, 879-887). All the other nuclear receptors have five amino acids in the same region.

The hNUC1B protein, unlike hPPARα, is not transcriptionally activated in HepG2 or CV-1 cells by CFA, ETYA or WY-14,643 (Fig. 1B, C). The slight activation seen in the absence of transfected receptor is probably due to the endogenous PPARs in the cell line utilized. This has also been observed by Schmidt, A., N. Endo, S. J. Rutledge, R. Vogel, D. Shinar, and G. A. Rodan. (1992) Mol. Endocrinol. 6, 1634-1641 with hNUC1 and certain fatty acids. This data suggests the absence of a PPAR activator inducible transactivation function in hNUC1B.

Transfected hNUC1B, however, did decrease the response from the endogenous PPARs. This suggested that hNUC1B may act as a repressor of hPPAR function. Figure 4 shows that increasing ratio of hPPARα to hNUC1B overcame the repression by hNUC1B.

Example 3. Screening for hNUC1B Inhibitors with Co-transfection Assay

Increasing amounts of hNUC1B plasmid were co-transfected with a constant amount of hPPARα expressing plasmid into cells. Figure 2 shows a strong dose dependent repression of hPPARα activity by hNUC1B in the presence of CFA. Repression was 85% with 0.1 μg of cotransfected hNUC1B plasmid. Repression by hNUC1 was also observed on the rat PPAR (Gottlicher, M., Widmar, E., Li, Q., and Gustafsson, J. A. (1992) Proc. Natl. Acad. Sci. USA. 89, 4653-4657) and on hPPARα in the presence of ETYA and WY-14,643.

To determine whether hNUC1B is a specific repressor

of hPPARα, we tested the effect of hNUC1B on other members of the steroid receptor family (Fig. 3 A-C).

hNUC1B has minimal effect on activation of ER and RARα by their respective ligands. hNUC1B does not repress RXRα in the absence of CFA and only 25% repression was detected in its presence.

However, with hTRβ1 and a palindromic TRE, 65% repression by hNUC1 was observed in the absence of CFA (Fig. 3 D). Repression increased to 75% in the presence of CFA. Repression was also observed with hTRα, although to a lesser degree.

Therefore, hNUC1B is not a general transcription repressor, but a dominant negative repressor of hPPARα and hTR. Repression occurred in the absence of clofibric acid, but was enhanced in its presence.

In order to screen for agents that relieve the repression PPARα and TR activity by hNUC1B, PPARα and hNUC1B or TR and hNUC1B expressing plasmids will be contransfected into CV-1 (a monkey kidney cell line) or HepG2 (a human liver cell line) cells along with a reporter containing PPAR or TR binding elements (such as PPREs, or TREs) in the presence of a PPAR activator (e.g., clofibiric acid, WY-14,643) or a TR activator (e.g., LT3).

Clofibric acid or LT3 normally activate their respective receptors and will therefore give a strong signal. In the presence of hNUC1B the signal will be very weak because of repression of these receptors by hNUC1B. We will add compounds to the transfected cells at various concentrations and select those that relieve the repression by hNUC1B.

The above screening strategy will also be followed in a yeast based assay with appropriate vectors and reporters.

Example 4. Screening for hNUC1B Inhibitors by Gel Retardation Assay

Gel retardation assays showed that hNUC1B binds to

a PPAR element, PPRE. With hNUC1B or hRXRα alone, weak retarded complexes were seen (Fig. 5, lanes 1 and 2). Addition of RXRα enhances binding of hNUC1B (lane 3), demonstrating co-operative binding of hNUC1B and hRXRα to the PPRE. Similar results have been observed with mPPARα and rPPAR (Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A., and Evans, R. M. (1992) Nature (London) **358**, 771-774; and Isseman, I., Prince, R.A., Tugwood, J.D., and Green, S. (1993) Biochemie **75**, 251-256. Gearing, K. L., Gottlicher, M., Teboul, M., Widmark, E., and Gustafsson., (1993) J. Proc. Natl. Acad. Sci. USA. **90**, 1440-1444).

Since hNUC1B could be repressing the activation of PPARα or TR simply by binding to the DNA sequences normally bound by these receptors, we will screen for compounds that prevent formation of a hNUC1B-PPRE complex by adding them during the binding reaction. Similar reaction assays will be done with TRα and TRβ.

Example 5. Screening for hNUC1B Inhibitors with Immuno-precipitation assay

hNUC1B may simply dimerize with PPARα or TR to form an inactive heterodimer. To screen for agents that relieve the repression PPARα and TR activity by hNUC1B, we will mix labeled hNUC1B with unlabeled TR or PPARα. TR or PPARα specific antibodies will then be used to immunoprecipitate the hNUC1B-TR or hNUC1B-PPARα complexes respectively. Test compounds will be added to this mix and only those that disrupt the formation of these hetero-dimers will be selected. These compounds will then be further tested by the methods described above to see if they relieve repression of PPAR and TR by hNUC1B.

Example 6. Toxicity-testing of Putative NUC Inhibitors

Methods are provided for determining whether an agent active in any of the methods listed above has little or no effect on healthy cells. Such agents are then

formulated in a pharmaceutically acceptable buffer or in buffers useful for standard animal tests.

By "pharmaceutically acceptable buffer" is meant any buffer which can be used in a pharmaceutical composition prepared for storage and subsequent administration, which comprise a pharmaceutically effective amount of an agent as described herein in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985). Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. Id. at 1449. In addition, antioxidants and suspending agents may be used. Id.

A. Additional screens for Toxicity: Method 1

Putative NUC inhibitors are assessed for toxicity to cultured human cells. This assessment is based on the ability of living cells to reduce 2,3,-bis[2-methoxy-4-nitro-5-sulphonylphenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide] otherwise referred to as XTT (Paull et al., J. Heterocyl. Chem. 25:763-767 (1987); Weislow et al., (1989), J. Natl. Canc. Inst. 81:577).

Viable mammalian cells are capable of reductive cleavage of an N-N bond in the tetrazole ring of XTT to form XTT formazan. Dead cells or cells with impaired energy metabolism are incapable of this cleavage reaction. The extent of the cleavage is directly proportional to the number of living cells tested.

Cells from a human cell line such as HeLa cells are seeded at 10^3 per well in 0.1 ml of cell culture medium (Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum) in the wells of a 96 well microtiter plate. Cells are allowed to adhere to the plate by culture at 37° C in an atmosphere of 95% air, 5%

CO_2.

After overnight culture, solutions of test substances are added in duplicate to wells at concentrations that represent eight half-decade log dilutions. In parallel, the solvent used to dissolve the test substance is added in duplicate to other wells. The culture of the cells is continued for a period of time, typically 24 hours.

At the end of that time, a solution of XTT and a coupler (methylphenazonium sulfate) is added to each of the test wells and the incubation is continued for an additional 4 hours before the optical density in each of the wells is determined at 450 nm in an automated plate reader. Substances that kill mammalian cells, or impair their energy metabolism, or slow their growth are detected by a reduction in the optical density at 450 nm in a well as compared to a well which received no test substance.

B. Additional screens for Toxicity: Method 2

Putative NUC inhibitors are tested for cytotoxic effects on cultured human cell lines using incorporation of ^{35}S methionine into protein as an indicator of cell viability.

HeLa cells are grown in 96 well plates in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and 50μg/ml penicillin and streptomycin. Cells are initially seeded at 10^3 cells/well, 0.1 ml/well. Cells are grown for 48 hrs without exposure to the NUC inhibitor, then medium is removed and varying dilutions of the NUC inhibitor prepared in complete medium are added to each well, with control wells receiving no NUC inhibitor.

Cells are incubated for an additional 48-72 hrs. Medium is changed every 24 hrs and replaced with fresh medium containing the same concentration of the NUC inhibitors. Medium is then removed and replaced with complete medium without NUC inhibitor.

Cells are incubated for 24 hr in the absence of NUC inhibitor, then viability is estimated by the incor-

poration of ^{35}S into protein. Medium is removed, replaced with complete medium without methionine, and incubated for 30 min. Medium is again removed, and replaced with complete medium without methionine but containing 0.1 μCi/ml ^{35}S methionine. Cells are incubated for 3 hrs.

Wells are washed 3 times in PBS, then cells are permeabilized by adding 100% methanol for 10 min. Ice cold 10% trichloroacetic acid (TCA) is added to fill wells; plates are incubated on ice for 5 min. This TCA wash is repeated two more times. Wells are again washed in methanol, then air dried. 50μl of scintillation cocktail are added to each well and dried onto the wells by centrifugation. Plates are used to expose X ray film. Densitometer scanning of the autoradiogram, including wells without NUC inhibitor, is used to determine the dosage at which 50% of cells are not viable (ID_{50}) (Culture of Animal Cells. A manual of basic technique. (1987). R. Ian Freshney. John Wiley & Sons, Inc., New York).

Example 7. Pharmaceutical Formulations and Administration of NUC Inhibitor

The particular agent that affects hNUC1 activity and the pathological condition of interest can be administered to a patient either by themselves, or in pharmaceutical compositions where it is mixed with suitable carriers or excipient(s).

In treating a patient exhibiting a pathological condition induced or aggravated by the level of hNUC1 activity, a therapeutically effective amount of a agent or agents such as these is administered. By "therapeutically effective amount" is meant an amount that relieves (to some extent) one or more symptoms of the disease or condition in the patient. Additionally, by "therapeutically effective amount" is meant an amount that returns to normal, either partially or completely, physiological or biochemical parameters associated with or causative of a disease or condition.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). Each candidate compound is tested for its efficacy in relieving the repression of PPARα and TR by hNUC1 in cell lines, in animal models, and in controlled clinical studies using methods known to those skilled in the art and approved by the Food and Drug Administration, such as, but not limited to, those promulgated in the Federal Register 47 (no. 56) : 12558-12564, March 23, 1982. The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} as determined in cell culture (i.e., the concentration of the test compound which achieves a half-maximal disruption of the protein complex, or a half-maximal inhibition of the cellular level and/or activity of a complex component). Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by HPLC.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view

of the patient's condition. (See e.g. Fingl et al., in The Pharmacological Basis of Therapeutics, 1975, Ch. 1 p. 1). It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administrated dose in the management of the cardiovascular disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

Depending on the specific conditions being treated, such agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA (1990). Suitable routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharma-

ceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If

desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

All publications referenced are hereby incorporated by reference herein, including the nucleic acid sequences and amino acid sequences listed in each publication.

Other embodiments are within the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Mukherjee, Ranjan

(ii) TITLE OF INVENTION: SCREENING FOR NUC INHIBITOR

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:

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(C)	CITY:	Los Angeles
(D)	STATE:	California
(E)	COUNTRY:	U.S.A.
(F)	ZIP:	90071-2066

(v) COMPUTER READABLE FORM:

(A)	MEDIUM TYPE:	3.5" Diskette 1.44 Mb storage
(B)	COMPUTER:	IBM Compatible
(C)	OPERATING SYSTEM:	IBM P.C. DOS 5.0
(D)	SOFTWARE:	Word Perfect 5.1

(vi) CURRENT APPLICATION DATA:

(A)	APPLICATION NUMBER:	08/270,635
(B)	FILING DATE:	July 1, 1994
(C)	CLASSIFICATION:	514

(vii) PRIOR APPLICATION DATA:

Prior applications total,
including application

described below: 2

(A) APPLICATION NUMBER: 08/141,500
(B) FILING DATE: October 22, 1993
(A) APPLICATION NUMBER: 08/143,215
(B) FILING DATE: October 25,1993

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Warburg, Richard J.
(B) REGISTRATION NUMBER: 32,327
(C) REFERENCE/DOCKET NUMBER: 207/200

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (213) 489-1600
(B) TELEFAX: (213) 955-0440
(C) TELEX: 67-3510

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1326 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATGGAGCAGC CACAGGAGGA AGCCCCTGAG GTCCGGGAAG AGGAGGAGAA AGAGGAAGTG 60

GCAGAGGCAG AAGGAGCCCC AGAGCTCAAT GGGGGACCAC AGCATGCACT TCCTTCCAGC 120

AGCTACACAG ACCTCTCCCG GAGCTCCTCG CCACCCTCAC TGCTGGACCA ACTGCAGATG 180

GGCTGTGACG GGGCCTCATG CGGCAGCCTC AACATGGAGT GCCGGGTGTG CGGGGACAAG 240

GCATCGGGCT TCCACTACGG TGTTCATGCA TGTGAGGGGT GCAAGGGCTT CTTCCGTCGT 300

ACGATCCGCA TGAAGCTGGA GTACGAGAAG TGTGAGCGCA GCTGCAAGAT TCAGAAGAAG 360

AACCGCAACA AGTGCCAGTA CTGCCGCTTC CAGAAGTGCC TGGCACTGGG CATGTCACAC 420

AACGCTATCC GTTTTGGTCG GATGCCGGAG GCTGAGAAGA GGAAGCTGGT GGCAGGGCTG 480

ACTGCAAACG AGGGGAGCCA GTACAACCCA CAGGTGGCCG ACCTGAAGGC CTTCTCCAAG 540

CACATCTACA ATGCCTACCT GAAAAACTTC AACATGACCA AAAAGAAGGC CCGCAGCATC 600

CTCACCGGCA AAGCCAGCCA CACGGCGCCC TTTGTGATCC ACGACATCGA GACATTGTGG 660

CAGGCAGAGA AGGGGCTGGT GTGGAAGCAG TTGGTGAATG GCCTGCCTCC CTACAAGGAG 720

ATCAGCGTGC ACGTCTTCTA CCGCTGCCAG TGCACCACAG TGGAGACCGT GCGGGAGCTC 780

ACTGAGTTCG CCAAGAGCAT CCCCAGCTTC AGCAGCCTCT TCCTCAACGA CCAGGTTACC 840

CTTCTCAAGT ATGGCGTGCA CGAGGCCATC TTCGCCATGC TGGCCTCTAT CGTCAACAAG 900

GACGGGCTGC TGGTAGCCAA CGGCAGTGGC TTTGTCACCC GTGAGTTCCT GCGCAGCCTC 960

CGCAAACCCT TCAGTGATAT CATTGAGCCT AAGTTTGAAT TTGCTGTCAA GTTCAACGCC 1020

CTGGAACTTG ATGACAGTGA CCTGGCCCTA TTCATTGCGG CCATCATTCT GTGTGGAGAC 1080

CGGCCAGGCC TCATGAACGT TCCACGGGTG GAGGCTATCC AGGACACCAT CCTGCGTGCC 1140

CTCGAATTCC ACCTGCAGGC CAACCACCCT GATGCCCAGT ACCTCTTCCC CAAGCTGCTG 1200

CAGAAGATGG CTGACCTGCG GCAACTGGTC ACCGAGCACG CCCAGATGAT GCAGCGGATC 1260

AAGAAGACCG AAACCGAGAC CTCGCTGCAC CCTCTGCTCC AGGAGATCTA CAAGGACATG 1320

TACTAA 1326

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 441 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION : SEQ ID NO: 2

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Lys Glu Glu Val Ala Glu Ala Glu Gly Ala Pro Glu Leu Asn Gly Gly
            20                  25                  30

Pro Gln His Ala Leu Pro Ser Ser Ser Tyr Thr Asp Leu Ser Arg Ser
        35                  40                  45

Ser Ser Pro Pro Ser Leu Leu Asp Gln Leu Gln Met Gly Cys Asp Gly
    50                  55                  60

Ala Ser Cys Gly Ser Leu Asn Met Glu Cys Arg Val Cys Gly Asp Lys
65                  70                  75                  80

Ala Ser Gly Phe His Tyr Gly Val His Ala Cys Glu Gly Cys Lys Gly
                85                  90                  95

Phe Phe Arg Arg Thr Ile Arg Met Lys Leu Glu Tyr Glu Lys Cys Glu
            100                 105                 110

Arg Ser Cys Lys Ile Gln Lys Lys Asn Arg Asn Lys Cys Gln Tyr Cys
        115                 120                 125

Arg Phe Gln Lys Cys Leu Ala Leu Gly Met Ser His Asn Ala Ile Arg
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130 135 140

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145 150 155 160

Thr Ala Asn Glu Gly Ser Gln Tyr Asn Pro Gln Val Ala Asp Leu Lys
165 170 175

Ala Phe Ser Lys His Ile Tyr Asn Ala Tyr Leu Lys Asn Phe Asn Met
180 185 190

Thr Lys Lys Lys Ala Arg Ser Ile Leu Thr Gly Lys Ala Ser His Thr
195 200 205

Ala Pro Phe Val Ile His Asp Ile Glu Thr Leu Trp Gln Ala Glu Lys
210 215 220

Gly Leu Val Trp Lys Gln Leu Val Asn Gly Leu Pro Pro Tyr Lys Glu
225 230 235 240

Ile Ser Val His Val Phe Tyr Arg Cys Gln Cys Thr Thr Val Glu Thr
245 250 255

Val Arg Glu Leu Thr Glu Phe Ala Lys Ser Ile Pro Ser Phe Ser Ser
260 265 270

Leu Phe Leu Asn Asp Gln Val Thr Leu Leu Lys Tyr Gly Val His Glu
275 280 285

Ala Ile Phe Ala Met Leu Ala Ser Ile Val Asn Lys Asp Gly Leu Leu
290 295 300

Val Ala Asn Gly Ser Gly Phe Val Thr Arg Glu Phe Leu Arg Ser Leu
305 310 315 320

Arg Lys Pro Phe Ser Asp Ile Ile Glu Pro Lys Phe Glu Phe Ala Val
325 330 335

Lys Phe Asn Ala Leu Glu Leu Asp Asp Ser Asp Leu Ala Leu Phe Ile
340 345 350

Ala Ala Ile Ile Leu Cys Gly Asp Arg Pro Gly Leu Met Asn Val Pro
355 360 365

Arg Val Glu Ala Ile Gln Asp Thr Ile Leu Arg Ala Leu Glu Phe His
370 375 380

Leu Gln Ala Asn His Pro Asp Ala Gln Tyr Leu Phe Pro Lys Leu Leu
385 390 395 400

Gln Lys Met Ala Asp Leu Arg Gln Leu Val Thr Glu His Ala Gln Met
405 410 415

Met Gln Arg Ile Lys Lys Thr Glu Thr Glu Thr Ser Leu His Pro Leu
420 425 430

Leu Gln Glu Ile Tyr Lys Asp Met Tyr
435 440

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AGGTCACAGG AGGTCA 16

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCCGAACGTG ACCTTTGTCC TGGTCC 26

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTAGCGATAT CATGACCTTT GTCCTAGGCC TC 32

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

CTAGGAGGCC TAGGACAAAG GTCATGATAT CG 32

WHAT IS CLAIMED IS:

1. Method for screening for a therapeutic agent for treatment of a pathological condition affected by the level of NUC protein activity, comprising the step of screening for an inhibitor of NUC protein activity.

2. The method of claim 1, wherein said screening comprising the steps of:

contacting a potential agent with a system comprising a NUC protein and a PPARα protein, wherein the activity of said PPARα protein is repressed or reduced by said NUC protein;

measuring the activity of said PPARα protein; wherein an increase in the activity of said PPARα protein compared to the activity in the absence of said agent is an indication that said agent is potentially useful for treatment of said condition.

3. The method of claim 2, wherein said system further comprises a reporter gene whose expression is activated by said PPARα protein activity; and said PPARα protein activity is measured by the expression level of said reporter gene.

4. The method of claim 3, wherein said system comprises a cell.

5. The method of claim 4, wherein said NUC protein is expressed from a vector transfected into said cell.

6. The method of claim 4, wherein said PPARα protein is expressed from a vector transfected into said cell.

7. The method of claim 4, wherein said reporter gene is transfected into said cell in a vector.

8. The method of claim 3, wherein said system comprises an extract of a cell.

9. The method of claim 3, wherein said system further comprises a PPAR activator.

10. The method of claim 9, wherein said activator is selected from a group consisting of CFA, ETYA, and WY-14, 643.

11. The method of claim 3, wherein said reporter gene comprises a PPRE element.

12. The method of claim 2, wherein said NUC protein consists of a hNUC1B protein.

13. The method of claim 2, wherein said NUC protein consists of a hNUC1 protein.

14. The method of claim 1, wherein said screening comprising the steps of:

contacting a potential agent with a system comprising a NUC protein and a TR protein, wherein the activity of said TR protein is repressed or reduced by said NUC protein;

measuring the activity of said TR protein; wherein an increase in the activity of said TR protein compared to the activity in the absence of said agent is an indication that said agent is potentially useful for treatment of said condition.

15. The method of claim 14, wherein said system further comprises a reporter gene whose expression is activated by said TR protein activity; and said TR protein activity is measured by the expression level of said reporter gene.

16. The method of claim 15, wherein said system comprises a cell.

17. The method of claim 16, wherein said NUC protein is expressed from a vector transfected into said cell.

18. The method of claim 16, wherein said TR protein is expressed from a vector transfected into said cell.

19. The method of claim 16, wherein said reporter gene is transfected into said cell in a vector.

20. The method of claim 15, wherein said system comprises an extract of a cell.

21. The method of claim 15, wherein said system further comprises a TR activator.

22. The method of claim 21, wherein said activator is selected from a group consisting of LT3, LT4 and Triac.

23. The method of claim 15, wherein said reporter gene comprises a TRE element.

24. The method of claim 14, wherein said NUC protein consists of a hNUC1B protein.

25. The method of claim 14, wherein said NUC protein consists of a hNUC1 protein.

26. The method of claim 1, wherein said screening comprising the steps of:

contacting a potential agent with a system comprising a NUC protein and a nucleic acid comprising a PPRE element, wherein said NUC protein binds to said nucleic acid to form a complex;

measuring the level of binding between said NUC protein and said nucleic acid; wherein a reduction in the binding compared to the binding in the absence of said agent is an indication that said agent is potentially useful for treatment of said condition.

27. The method of claim 26, wherein said measuring comprises determining the ratio of said nucleic acid in said complex and said nucleic without said complex.

28. The method of claim 26, wherein said NUC protein consists of a hNUC1B protein.

29. The method of claim 26, wherein said NUC protein consists of a hNUC1 protein.

30. The method of claim 1, wherein said screening comprising the steps of:

contacting a potential agent with a system comprising a NUC protein and a PPARα protein, wherein said NUC protein binds to said PPARα protein to form a NUC-PPARα complex;

measuring the level of said NUC-PPARα complex; wherein a reduction in the level of said NUC-PPARα complex compared to the level in the absence of said agent is an indication that said agent is potentially useful for treatment of said condition.

31. The method of claim 30, wherein said system further comprises a PPARα antibody, and said measuring comprises determining the level of said NUC-PPARα complex precipitated by said PPARα antibody.

32. The method of claim 31, wherein said NUC protein is labeled with a radioactive element.

33. The method of claim 30, wherein said NUC

protein consists of a hNUC1B protein.

34. The method of claim 30, wherein said NUC protein consists of a hNUC1 protein.

35. The method of claim 1, wherein said screening comprising the steps of:

contacting a potential agent with a system comprising a NUC protein and a TR protein, wherein said NUC protein binds to said TR protein to form a NUC-PPARα complex;

measuring the level of said NUC-TR complex; wherein a reduction in the level of said NUC-TR complex compared to the level in the absence of said agent is an indication that said agent is potentially useful for treatment of said condition.

36. The method of claim 35, wherein said system further comprises a TR antibody, and said measuring comprises determining the level of said NUC-TR complex precipitated by said TR antibody.

37. The method of claim 36, wherein said NUC protein is labeled with a radioactive element.

38. The method of claim 35, wherein said NUC protein consists of a hNUC1B protein.

39. The method of claim 35, wherein said NUC protein consists of a hNUC1 protein.

40. Method for treatment of a pathological condition affected by the level of NUC protein activity, comprising the step of providing an agent that represses or reduces said NUC protein activity.

41. The method of claim 40, wherein said

pathological condition is selected from a group consisting of hyperlipidemia, hypercholesteremia and hyperlipoproteinemia.

42. Purified nucleic acid comprising the nucleotide sequence shown in SEQ ID NO. 1.

43. A vector comprising said nucleic acid of claim 42.

44. Recombinant hNUC1B protein expressed from said nucleic acid of claim 42.

Figure 1

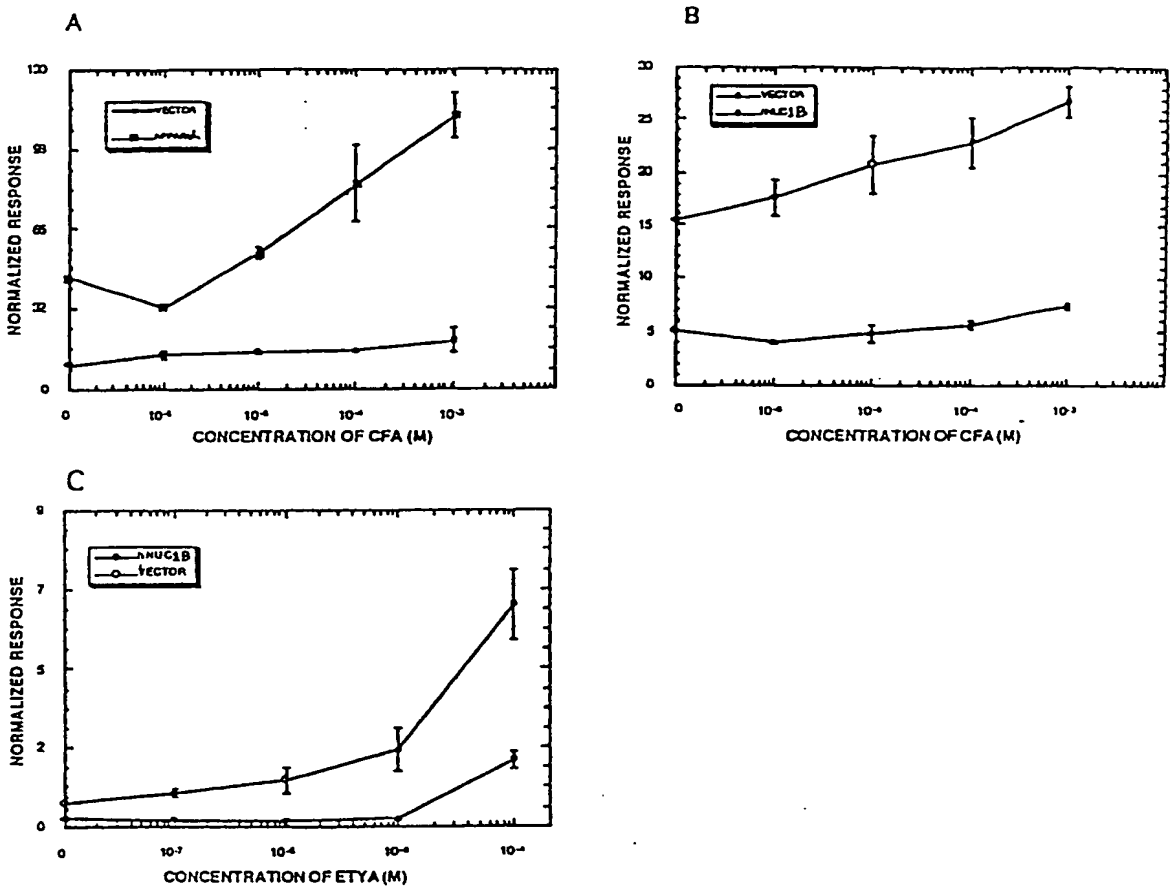
A
NORMALIZED RESPONSE
CONCENTRATION OF CFA (M)
B
NORMALIZED RESPONSE
CONCENTRATION OF CFA (M)
C
ΔNUC1B
VECTOR
NORMALIZED RESPONSE
CONCENTRATION OF ETYA (M)

Figure 2

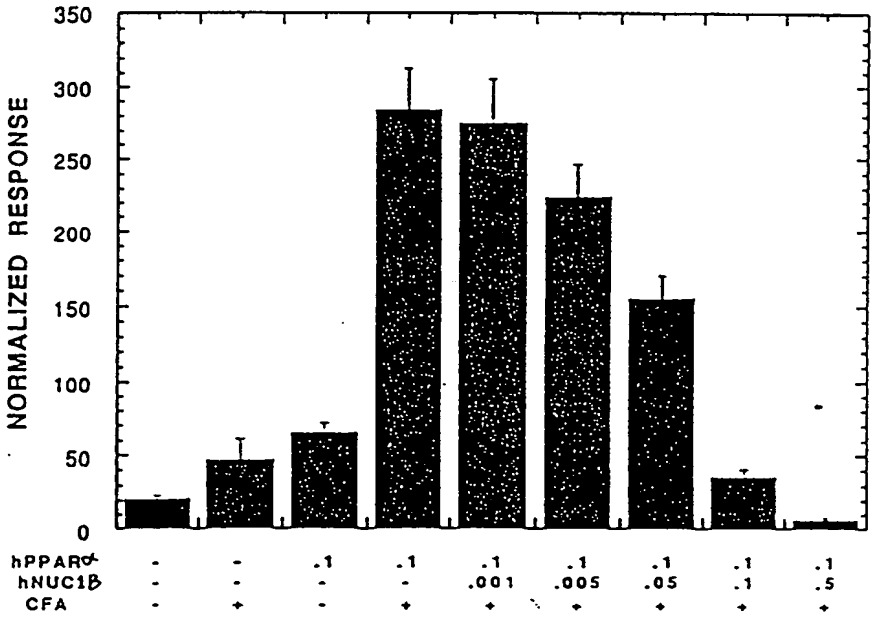
NORMALIZED RESPONSE
350
300
250
200
150
100
50
0
hPPARα - - .1 .1 .1 .1 .1 .1 .1
hNUC1β - - - - .001 .005 .05 .1 .5
CFA - + - + + + + + +

Figure 3

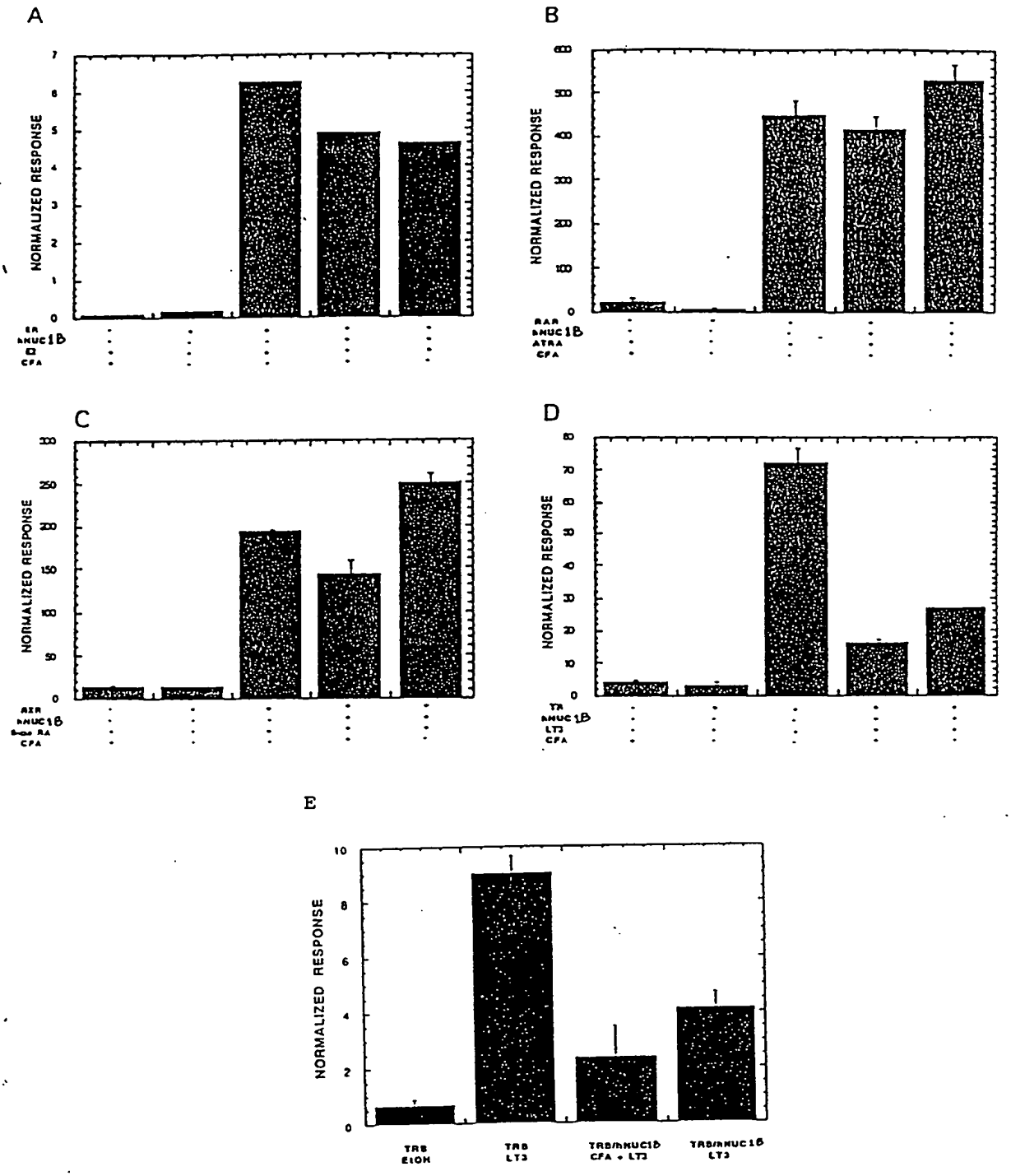
A
NORMALIZED RESPONSE
B
NORMALIZED RESPONSE
RAR
hNUC1B
ATRA
CPA
C
NORMALIZED RESPONSE
D
NORMALIZED RESPONSE
TR
hNUC1B
LT3
CPA
E
NORMALIZED RESPONSE
TRB EtOH
TRB LT3
TRB/hNUC1B CPA + LT3
TRB/hNUC1B LT3

Figure 4

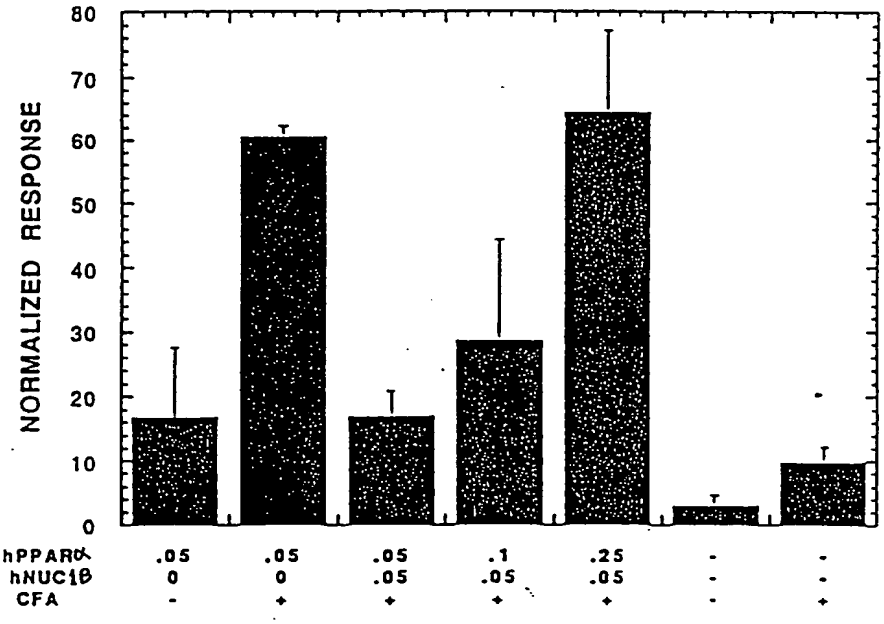
NORMALIZED RESPONSE
80
70
60
50
40
30
20
10
0
hPPARδ .05 .05 .05 .1 .25 - -
hNUC1β 0 0 .05 .05 .05 - -
CFA - + + + + - +

Figure 5

hNUC1β	+	+	+	−	−
hRXRα	−	+	−	+	+
mock transf.	−	−	+	−	+

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(74) Agents: WARBURG, Richard, J. et al.; Lyon & Lyon, First Interstate World Center, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).

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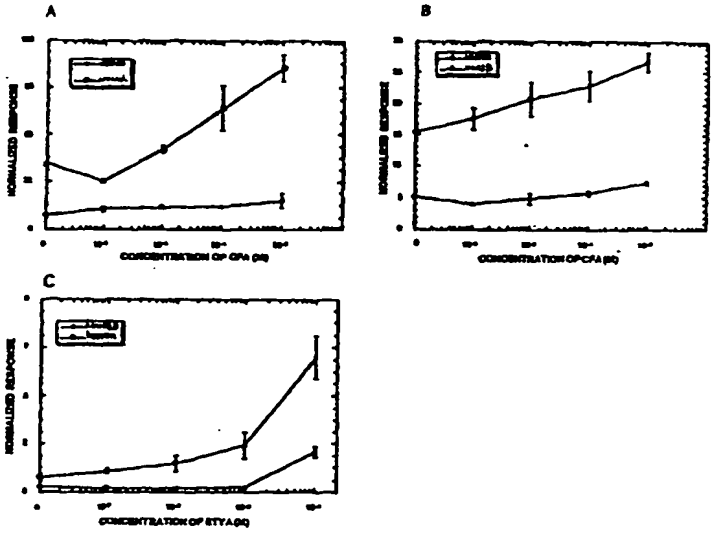

(57) Abstract

This invention provides methods for screening for agents useful for treatment of diseases and pathological conditions affected by the level of NUC protein activity. These agents reduce or relieve the repression of PPARα protein and TR protein transcription activation activity by NUC protein. The selected novel or unique agents can be used to treat hyperlipidemia, hypercholesteremia and hyperlipoproteinemia.

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INTERNATIONAL SEARCH REPORT

International Application No
PC./US 95/08328

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68 C12N15/12 C07K14/705 G01N33/74 G01N33/94

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 196, no. 2, 1993, NEW YORK NY USA, pages 671-677, XP000577084 F. CHEN ET AL.: "Identification of two mPPAR related receptors and evidence for the existence of five subfamily members " cited in the application see the whole document --- -/--	1-44

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2

Date of the actual completion of the international search	Date of mailing of the international search report
19 August 1996	30.08.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Authorized officer Van Bohemen, C

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International Application No
PC./US 95/08328

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MOLECULAR ENDOCRINOLOGY, vol. 6, no. 10, 1992, WASHINGTON DC USA, pages 749-784, XP000579262 A. SCHMIDT ET AL.: "Identification of a new member of the steroid hormone receptor superfamily that is activated by a peroxisome proliferator and fatty acids." cited in the application see the whole document ---	1-44
A,P	WO,A,95 11974 (LIGAND PHAMACEUTICALS INCORPORATED) 4 May 1995 see the whole document ---	1-44
A,P	WO,A,96 01317 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 18 January 1996 see the whole document ---	1-44
A,P	WO,A,95 18533 (UNIVERSITY OF PENNSYLVANIA) 13 July 1995 see page 14, line 9 - page 15, line 18 -----	1-44

2

INTERNATIONAL SEARCH REPORT

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International Application No
PC./US 95/08328

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9511974	04-05-95	AU-B-	8083194	22-05-95
		EP-A-	0724636	07-08-96
WO-A-9601317	18-01-96	AU-B-	2952695	25-01-96
WO-A-9518533	13-07-95	NONE		